

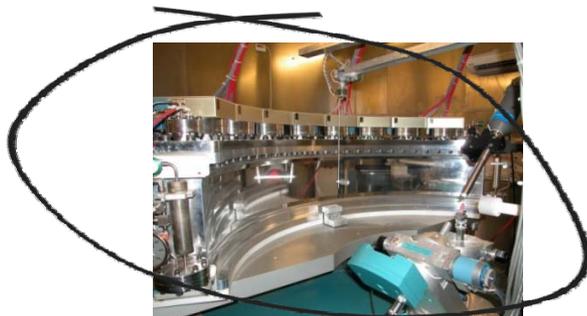
# Neutrons for Bioenergy

Paul Langan

## Proteomics and Protein Crystallography Team of the Bioenergy & Environment Science Group



Enzyme & Microbe Engineering

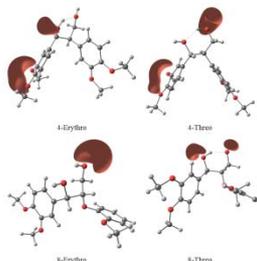


X-ray, Neutron & Computational Crystallography

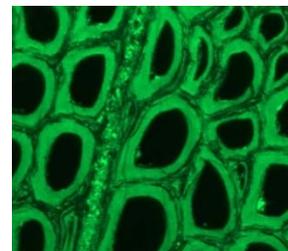


Mass Spectrometry

## Cellulosic Biofuels & Bioproducts



Theory & Modeling



Multiplatform Microspectroscopy

<http://biofuels.lanl.gov>

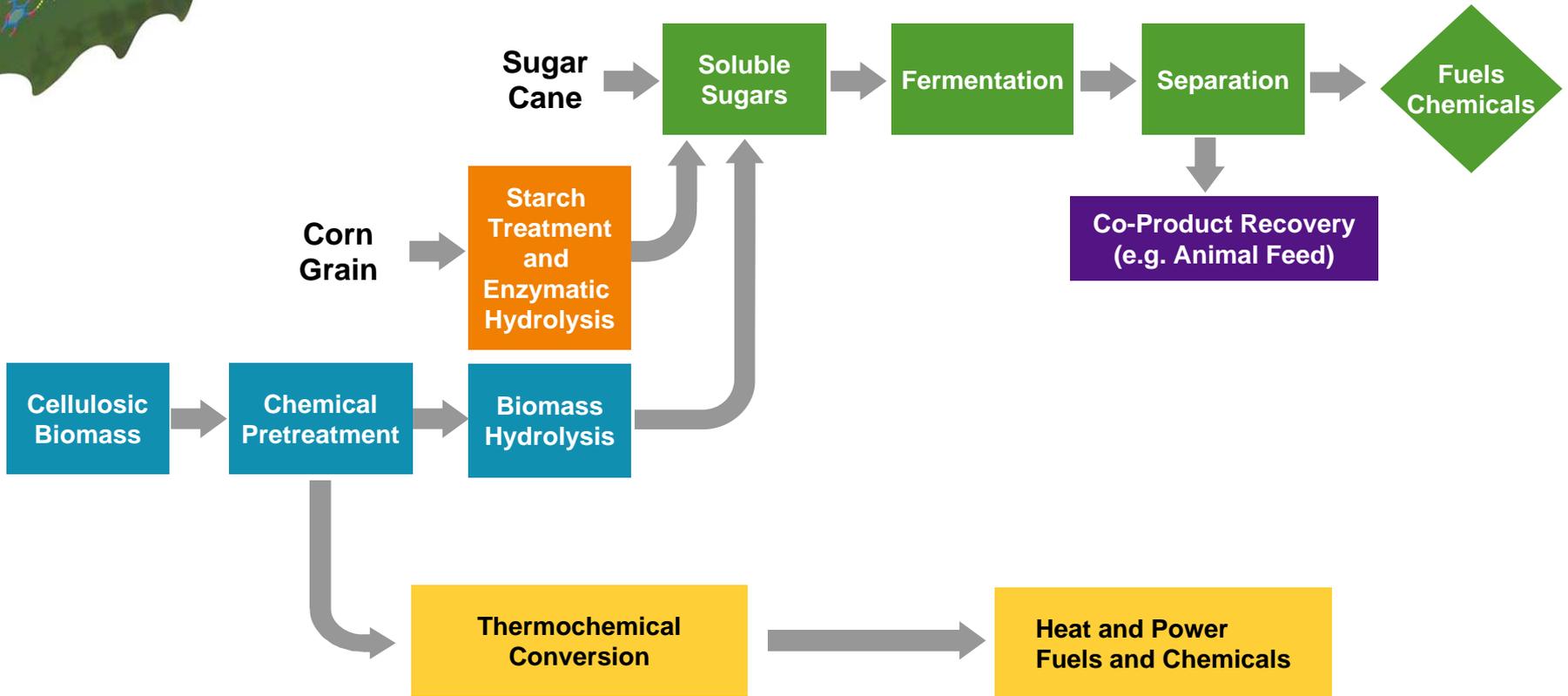
Funding: DOE-BER, DOE-OBP, USDA, DTRA, TMTI, DHS, UCOP, LDRD

# Fuels and chemicals from cellulosic biomass

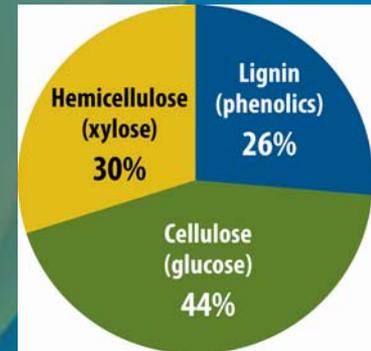
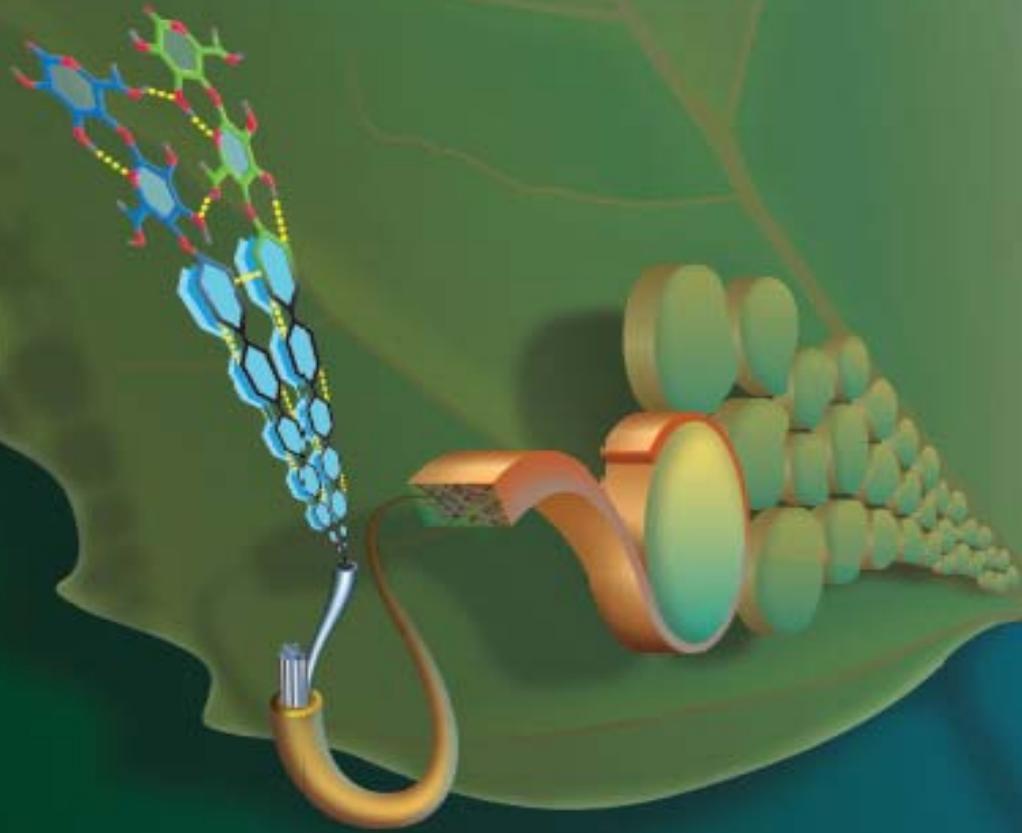
## Lignocellulosic Biomass Process

### Corn Starch Process

### Sugar Cane Process



# Biomass consists of cellulose, hemicellulose and lignin



The crystallinity of cellulose and its inaccessibility make it difficult to break down into fermentable sugars

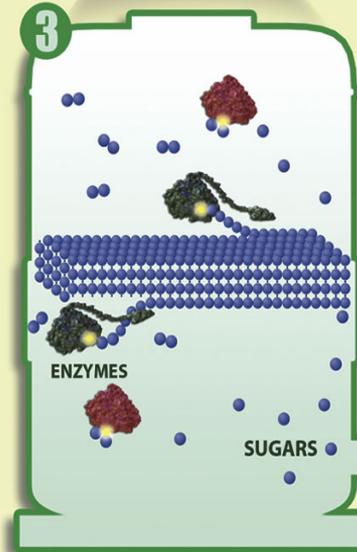
**A future, large-scale, cellulosic ethanol production facility.** (1) Biomass from trees, grasses, or agricultural wastes is harvested and delivered to the biorefinery. (2) Biomass is ground into small, uniform particles. Thermal or chemical pretreatment separates cellulose, a tough polymer of tightly bound sugar chains, from other biomass materials and opens up the cellulose surface to enzymatic attack. (3) A mix of enzymes is added to break down cellulose into simple sugars. (4) Microbes produce ethanol by fermenting sugars from cellulose and other biomass carbohydrates. (5) Ethanol is separated from water and other components of the fermentation broth and purified through distillation.



2

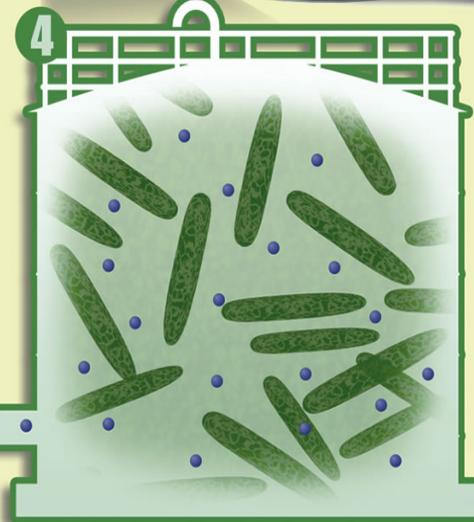
Biomass is cut into shreds and pretreated with heat and chemicals to make cellulose accessible to enzymes.

3



Enzymes break down cellulose chains into sugars.

4



Microbes ferment sugars into ethanol.

5

Ethanol is purified through distillation and prepared for distribution.

1

Biomass is harvested and delivered to the biorefinery.

# Problem: Cellulosic biomass pretreatment is expensive and inefficient

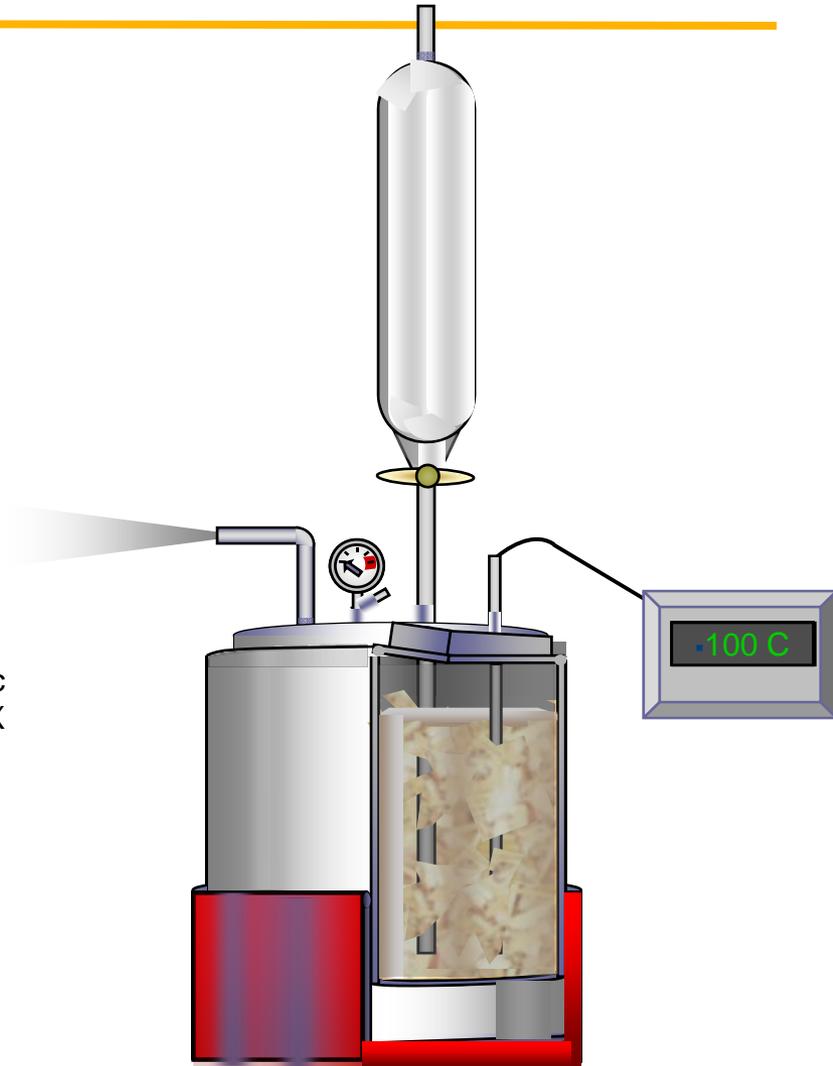
- We are investigating a variety of pretreatments of biomass in order to improve its catalytic conversion to various products.
- These include ionic liquids, thermochemical, mild acid, alkali mercerization, AFEX, and biological and oxidative pretreatment processes.
- Techniques used include time-resolved and multilength scale microscopy, diffraction, mass spec and theoretical approaches

## Advantages of AFEX over other processes:

- High Catalyst Recovery (>98%)
- Minimal Water Usage (3-20 fold lower)
- Minimal Biological Inhibitors Formed (e.g. furans)
- Flexible Feedstock (e.g. animal feed)

We are using neutron and crystallography, modeling, and enzymatic studies to understand structural changes that occur during 1) AFEX pretreatment 2) how catalysis depends on these changes.

**These insights are guiding the development of a new optimized process in collaboration with GLBRC (Shishir Chundawat).**



# Team and Capabilities

Microscopy

Crystallography

Theory

Degradomics

Bench and pilot scale

enzyme engineering

synthetic chemistry

Proteomics

Collaborations

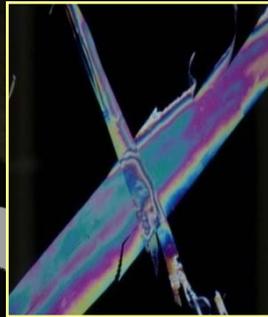
The screenshot shows a Microsoft Internet Explorer browser window displaying the website for Cellulosic BioFuels at Los Alamos National Laboratory. The browser's address bar shows the URL <http://biofuels.lanl.gov>. The website header includes the Los Alamos National Laboratory logo and navigation links for ABOUT LANL, NEWS, LIBRARY, and JOBS. Below the header, there is a navigation menu with Home, Projects, Publications, Partners, and Gallery. The main content area features a banner with the text "Cellulosic BioFuels" and "Los Alamos National Laboratory Innovation for Biofuels Consortium" alongside a graphic of a DNA double helix and a glowing molecular structure. Below the banner, a paragraph of text reads: "Biofuels are an alternative to conventional energy sources that increase our Nation's energy security by dramatically reducing our dependence on imported oil. Lignocellulosic biomass is an abundant renewable source that could be an important raw material for producing next generation biofuels. Many ways are being vigorously explored in laboratories throughout the world to convert lignocellulosic biomass into biofuels. Lignocellulosic biomass is the inedible fibrous material derived from plant cell walls, and its holocellulose component is composed of natural sugars that can be used for producing various fuels such as ethanol and butanol. The problem is the lack of energy-efficient and cost-effective processes for breaking up the plant cell wall and releasing these sugars". On the right side of the page, there is a sidebar with a "CAPABILITIES" section listing Microscopy, Crystallography, Theory, Proteomics, Enzymology, and Metabolomics. Below this is a "RELATED LINKS" section with a link to "CNLS Workshop".

# Providing the First Atomistic Structures of Cellulose

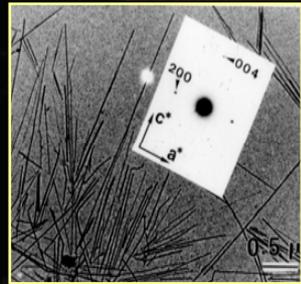
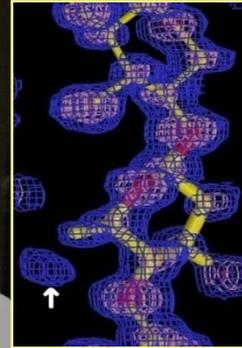


Natural sources of pure Cellulose allomorph

Prepare oriented films



X-ray Crystallography

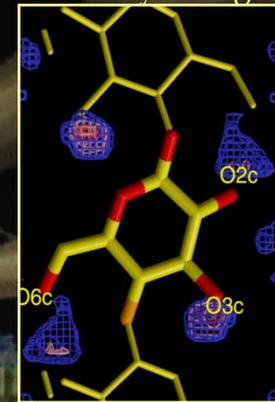


Purify highly crystalline Cellulose microfibrils

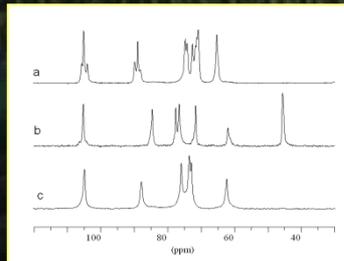


Crystallography

Neutron Crystallography



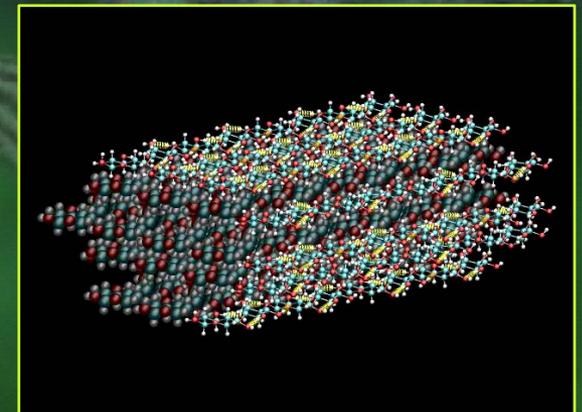
Cellulose Fiber



<sup>13</sup>C NMR CP/MAS

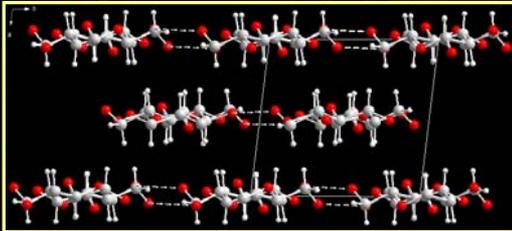


Modeling

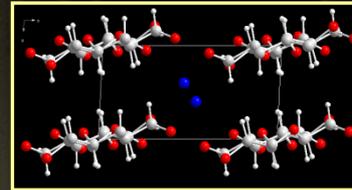


# Improving AFEX Pretreatment

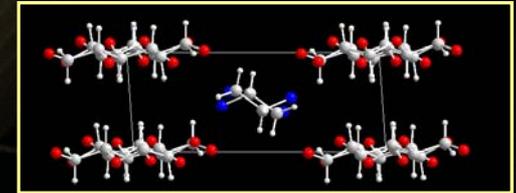
Results from neutron and X-ray crystallography and theory have lead to optimized conditions that improve hydrolysis of AFEX pretreated biomass and are leading to theoretical studies to create better celluloses



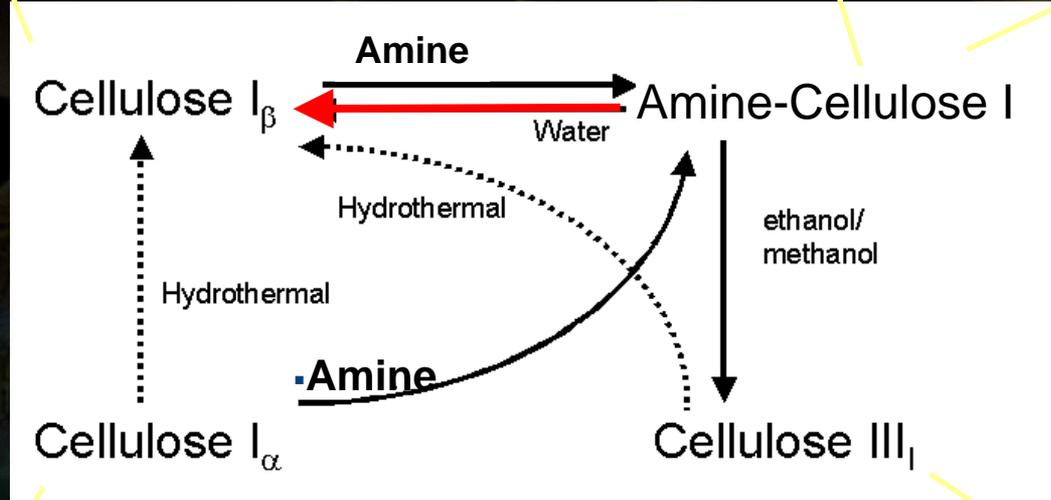
Nishiyama, Langan, Chanzy  
*J. Am. Chem. Soc.* 2002



Wada, Nishiyama, Langan  
*Macromolecules.* 2006



Wada, Heux, Nishiyama, Langan  
*Cellulose*, 2009

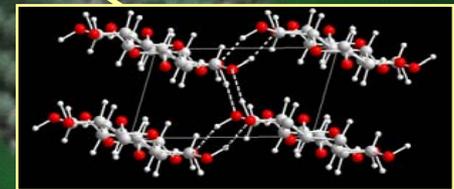
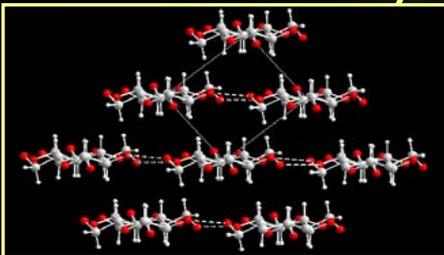


Wada, Nishiyama, Langan  
*J. Am. Chem.* 2010

Nishiyama, Sugiyama, Chanzy, Langan  
*J. Am. Chem. Soc.* 2003

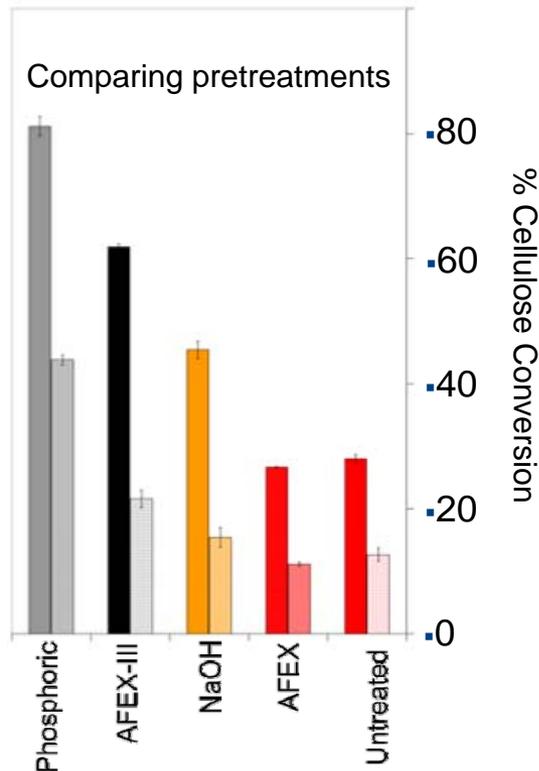
Wada, Chanzy, Nishiyama, Langan  
*Macromolecules*, 2004

Time-resolved X-ray microprobe studies reveal penetration of catalyst in biomass in situ and as its working (Wada, *et al.* Cellulose 2010)

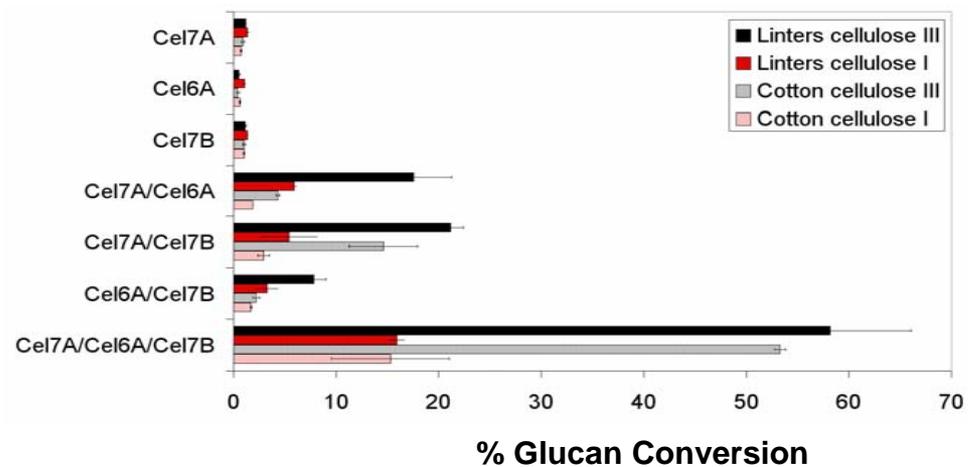


# Optimizing AFEX to incorporate conversion to cellulose III

Enzyme hydrolysis assays demonstrate greater efficiency of AFEX-III

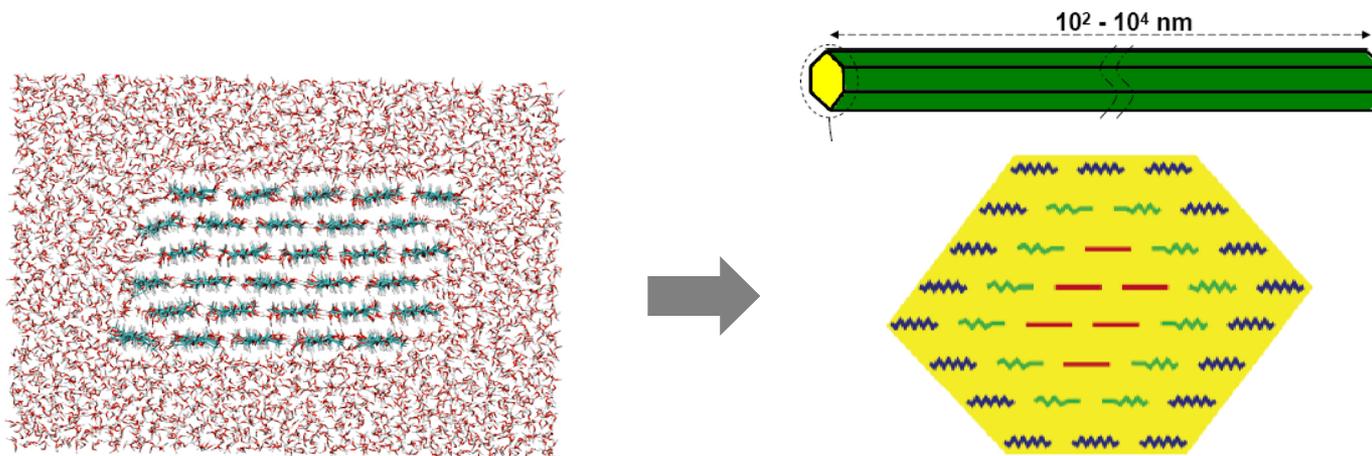


Developing biocatalyst cocktails that are tailored to AFEX-III



- Wada *et al. Biomacrol.*
- Chundawat *et al. Science*
- Chundawat *et al. PNAS*

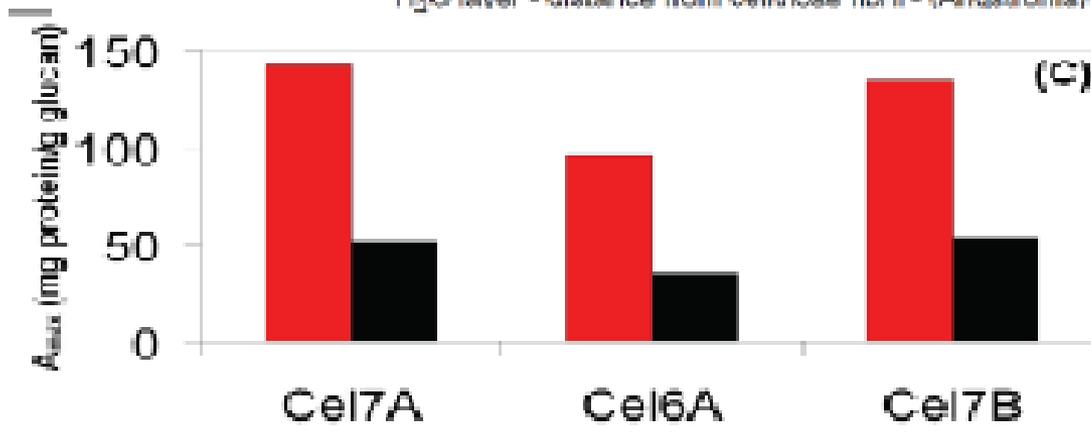
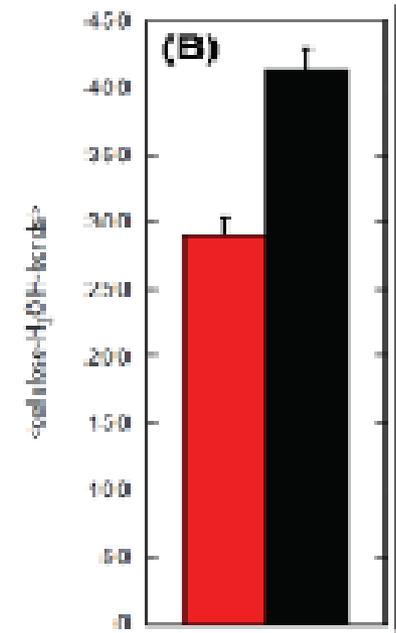
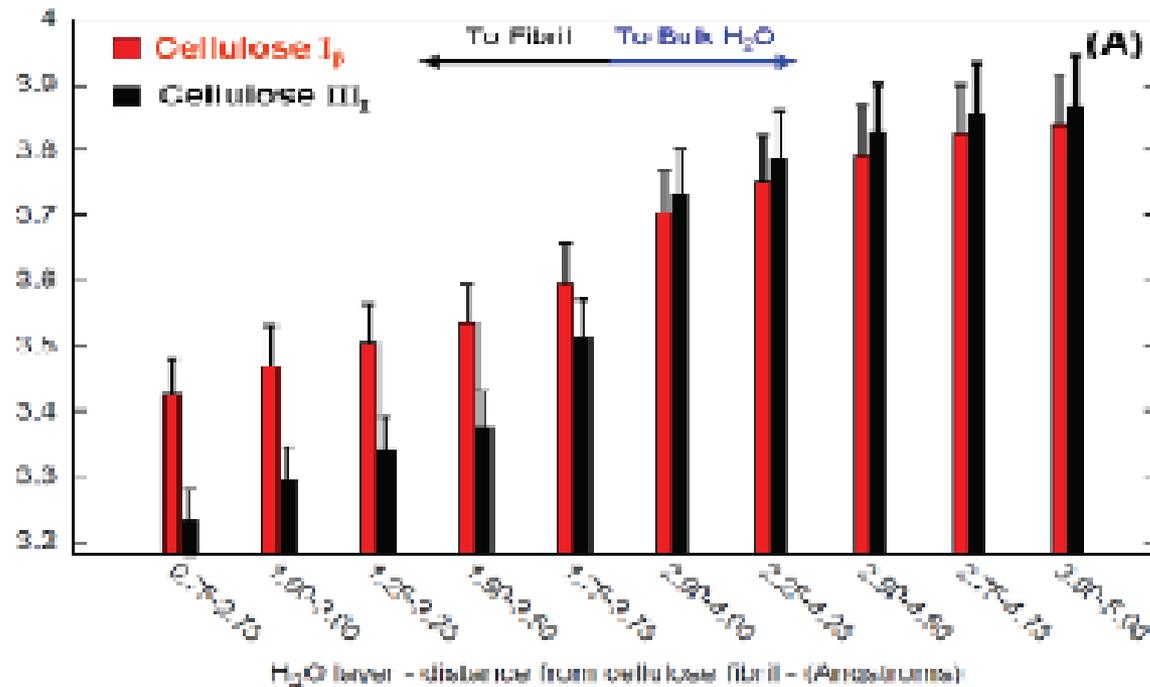
# Surface chains in cellulose III have similar conformations to solvated oligomers



Solvated cellulose octamer	<i>tg</i>	<i>gt</i>	<i>gg</i>
%	3.0	27.2	<b>69.8</b>
Cellulose I <sub>β</sub>	<i>tg</i>	<i>gt</i>	<i>gg</i>
Crystalline core			
%	<b>92.6</b>	4.7	2.7
Surface chains			
%	25.8	28.1	<b>46.1</b>
Cellulose III <sub>I</sub>	<i>tg</i>	<i>gt</i>	<i>gg</i>
Crystalline core			
%	11.8	<b>63.6</b>	24.6
Surface chains			
%	4.2	<b>52.3</b>	43.5

Shen, Langan, Gnanakaran, *J. Am. Chem.* 2009

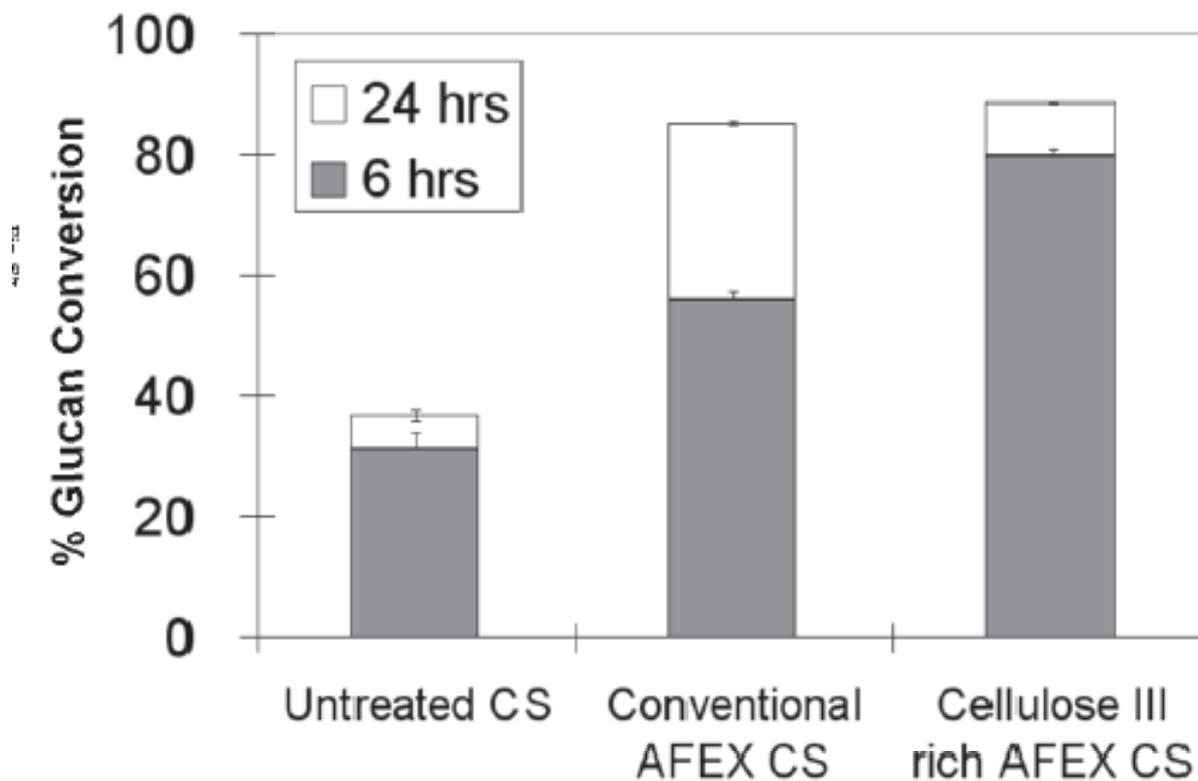
# Surface chains in cellulose III are hydrated like solvated oligomers



$$B = A_{max} * S * F / (K_d + F)$$

Where:  
 B – Bound protein  
 F – Free protein  
 A<sub>max</sub> – Maximum bound protein/g substrate  
 S – Amount of substrate available  
 K<sub>d</sub> – Association constant (1/K<sub>a</sub>)

## AFEX-III (NH<sub>3</sub> at 25°C for 2 hrs) conversion of corn stover



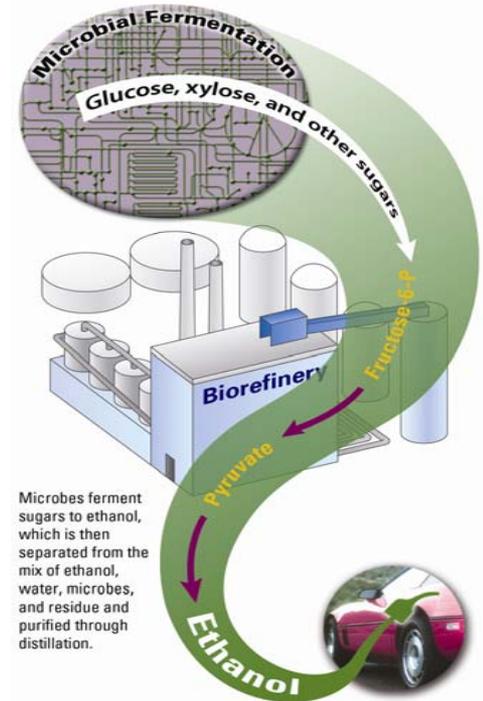
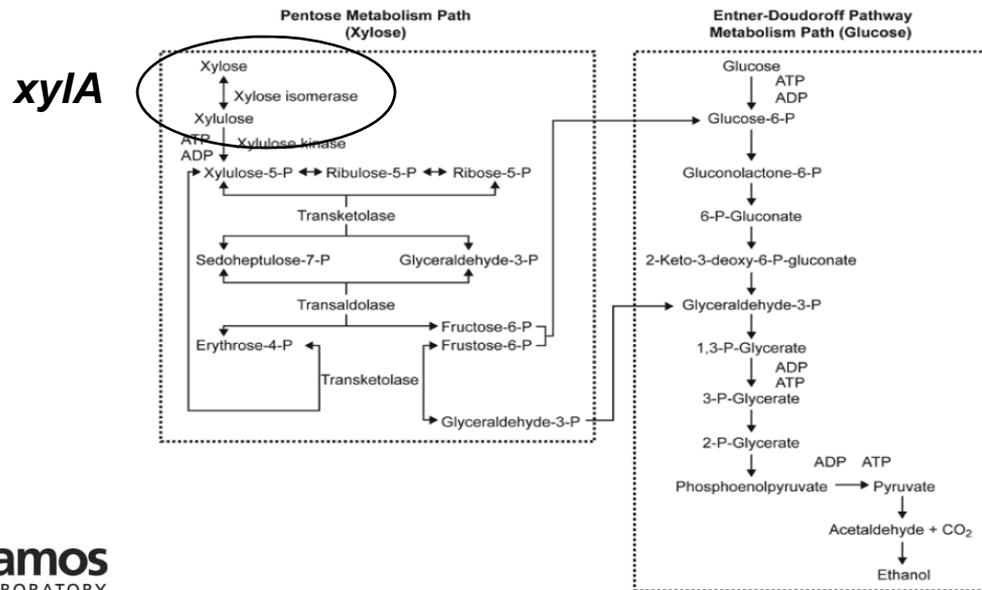
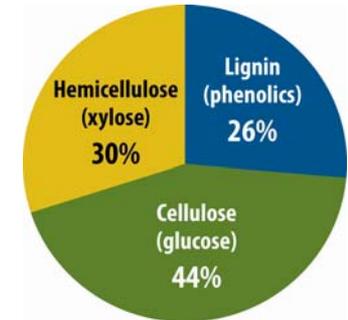
# Summary

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- We have provided atomistic details of the structure of cellulose (and biomass) and how it changes during pretreatment.
- Cellulose chains on the surface of cellulose fibrils have memory of their crystalline cores.
- Cellulose chains on the surface of cellulose III fibrils are similar to those in solution.
- An optimized AFEX-III pretreatment process significantly increases the conversion of corn stover.
- Co-optimization of substrate pretreatment and enzyme cocktails is essential.

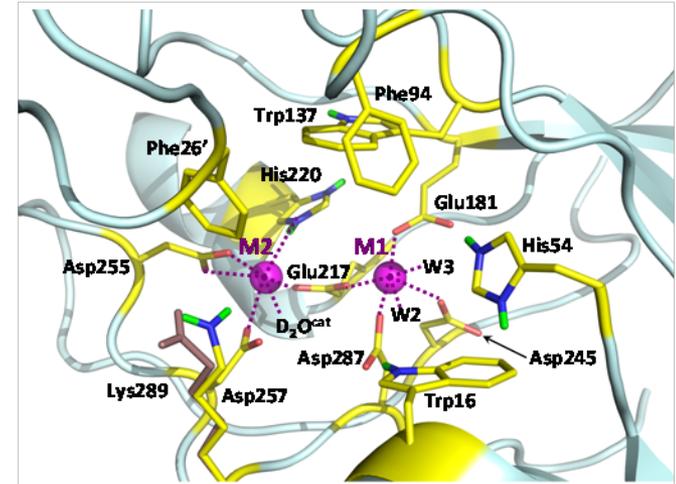
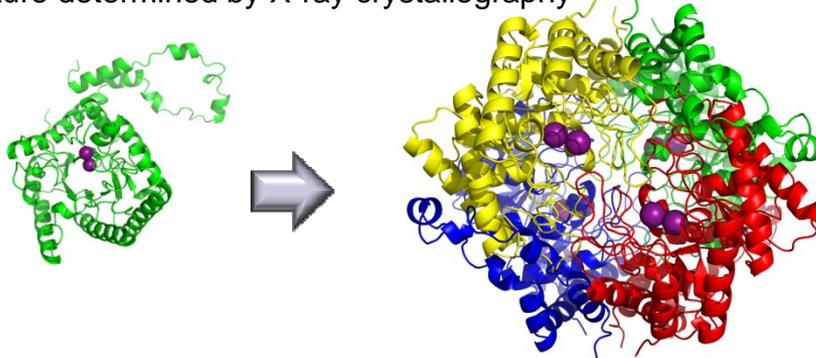
# Problem: Xylose from cellulosic biomass cannot be efficiently fermented to ethanol

- Cost-effective and sustainable production of biofuels from cellulosic biomass will require the use of all sugars
- Several different Metabolic Engineering and Systems Biology approaches are being taken to enable xylose fermentation in *S. cerevisiae*.
- We are determining the mechanism of **Xylose Isomerase** (XI) using neutron crystallography and quantum enzymology and reengineering for optimized catalysis in *S. cerevisiae*.

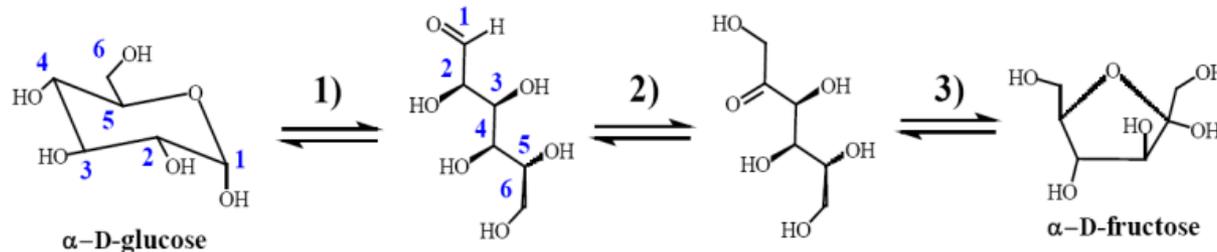


# Xylose Isomerase has been studied extensively by X-ray crystallography but its catalysis is still not understood

Structure determined by X-ray crystallography



H transfer occurs throughout this multistep catalytic process. H and its movement are invisible to X-rays.

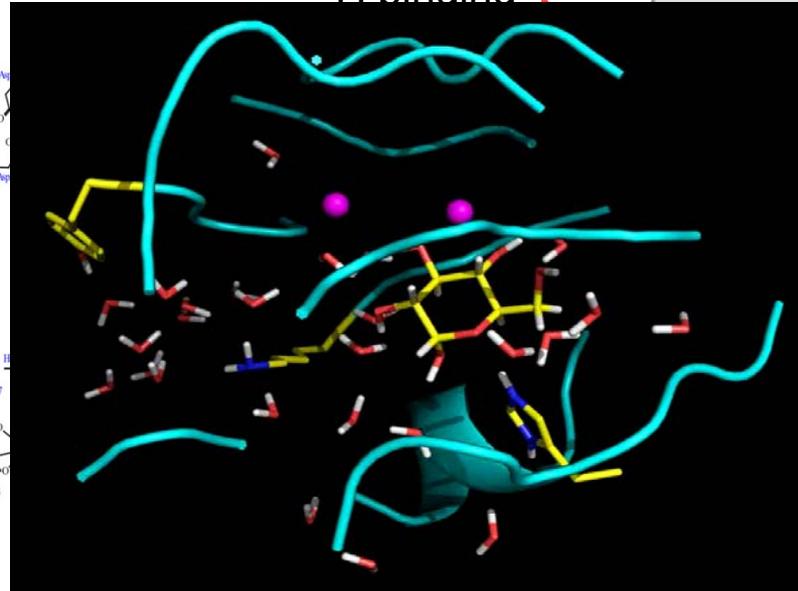
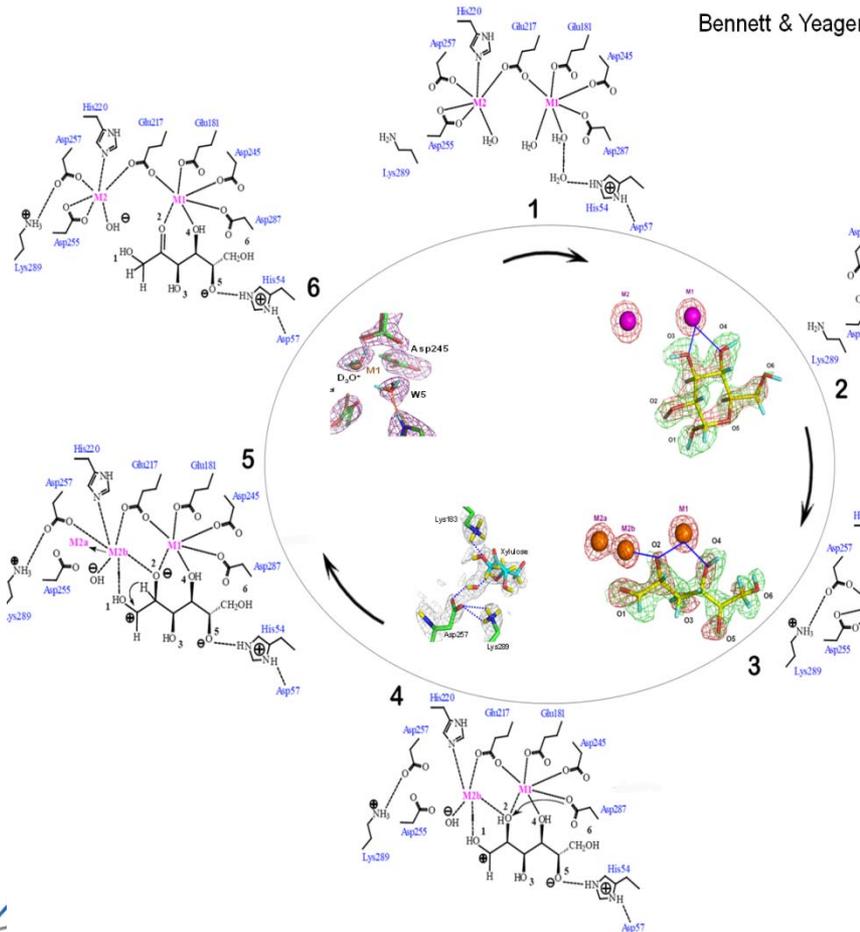


Many proposed mechanisms are consistent with X-ray structures but differ in the movement of H.

# Snapshot of different stages of the reaction reveal the possible movement of H during catalysis

Katz et al. *PNAS* 2006; Kovalevsky et al., *Biochem*, 2008; Kovalevsky et al. *Structure* 2010

Bennett & Yeager, *Structure*, 2010



Xylose Isomerase  
No Metal  
Low pH

Isomerase  
Metal  
Biological pH

Isomerase  
Metal  
 $+ \text{Cd}^{2+} \text{Ni}^{2+}$

Isomerase  
Metal  
Substrate

Isomerase  
Metal  
Linear Substrate

Xylose Isomerase  
Metal  
Product

Adams et al, *Acta Cryst* 2009; Afonine et al. *Acta Cryst* 2010; Fenn et al. *Structure*

# Quantum Enzymology (QuE) studies of xylose isomerase

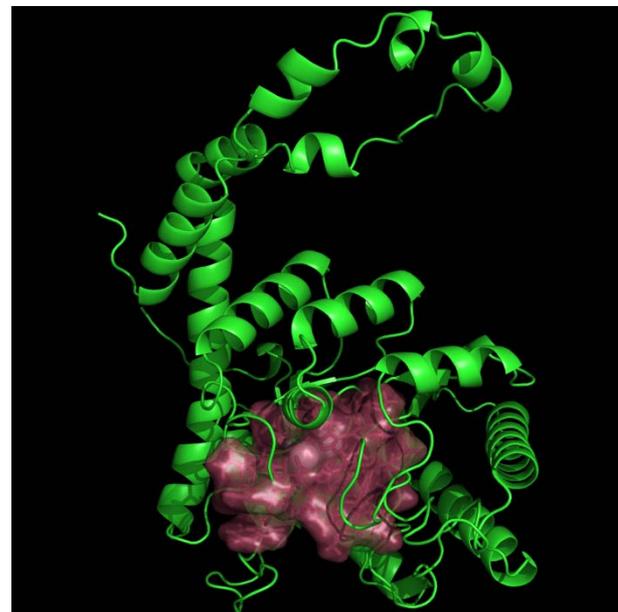
Neutron crystallography has provided snapshot of different stages of the reaction, revealing the possible movement of H during catalysis.

However, to fully characterize the reaction pathways, transition states, and energy barriers that connect these different stages a Quantum Mechanics approach is required. Conventional QM/MM boundary errors, don't work.

QuE combines neutron crystallography with unique LANL quantum chemistry algorithms to model of reactions catalyzed by enzymes.

Powerful new approach for

- understanding and predicting enzyme mechanism
- *in silico mutagenesis*
- *active site engineering.*



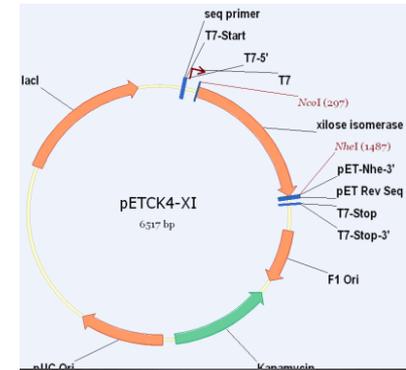
# Re-engineering Xylose Isomerase for lower pH and better $K_m$ xylose/ $K_i$ xylitol using results from PCS

## Initial Goals:

1. Redesign M1 site to prevent protonation and metal ejection at low pH
2. Lower  $pK_a$  of selected residues to enhance ring opening at low pH
3. Reduce  $K_m$  by optimizing water template for cyclic sugar
4. Increase  $K_{cat}$  for xylose and increase  $K_i$  for xylitol by careful optimization of transition state binding pocket using QuE.
5. Introduce improved *xyIA* into *S. Cerevisiae* for xylose utilization

## Progress:

1. Expression system (pETCk4) designed, *xyIA* with HisTAG has been expressed.
2. Easily purified and crystallized (library of ~100 clone generated).
3. Bioanalyzer assay for both glucose and xylose.
4. Using directed evolution we have already lowered pH of activity
5. NDA with Great Lakes Bioenergy Research Center for engineering of *S. Cerevisiae* (*Trey Sato*)



# Summary

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- Neutrons are being combined with theory & other experimental capabilities to provide new insights into biocatalysis
- These insights are guiding the design of improved and new synthetic biocatalysts.
- They are also guiding how biological substrates can be manipulated to enhance biocatalysis.
- These advances help address several major problems for DOE missions in energy and the environment.

The future development of the effective application of neutrons in biocatalysis will depend on several factors including (just personal opinion!).

1. Easy and simultaneous access to multiple complementary experimental platforms
2. Better access to large-scale modern computing facilities that allow complementary QM (QuE), MD and AB studies.
3. A new approach to bioengineering (synthetic biology) that allows co-optimization of different enzyme properties, and co-optimization of enzyme and microbe performance.
4. Co-optimization of biocatalyst and biosubstrate

# Acknowledgements

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**Proteomics and Protein Crystallography** Marc-Michael Blum (B8), David Edlin (B8), Zoe Fisher (B8), David Fox (B8), Srinivas Iyer (B8), Hannah Johnson (B8), Andy Koppish (B8), Andrey Kovalevsky (B8), Marat Mustyakimov (B8), Tim Sanchez (B8), Benno Schoenborn (B8), Mary Jo Waltman (B8)

**Xylose Isomerase** Andrey Kovalevsky (B8), Matt Challacombe (T1), Nick Bock (T1), Csaba Kiss (B9), Hannah Johnson (B8), Patsy Langan (B9), David Fox (B8), Jenny Glusker (Fox Chase Cancer Center), Leif Hanson (U. Toledo), Trey Sato (Great Lake Bioenergy Research Center).

**Carbonic anhydrase** Zoe Fisher (B8), Csaba Kiss (B9), Ryszard Michalcyk (B8), David Fox (B8), Andy Koppisch (B8), David Eldin (B8), Matt Challacombe (T1), Nick Bock (T1), Rob McKenna & David Silverman (U. Florida), National Alliance for Advanced Biofuels and Bioproducts

**OP detox & biosensors** Marc-Michael Blum (B8), Julian Chen (Frankfurt), Bob Williams (B7)

**AFEX** Shishir Chundawat (GLBRC), Bruce Dale (GLBRC), Masahisa Wada (Tokyo), Yoshi Nishiyama (CERMAV), Gnanakaran (T10), Giovanni Bellesia (T10)

**Neutron Computational Tools** Marat Mustyakimov (B8), Pavel Afonine (LBNL), Paul Adams (LBNL), Axel Brunger, Tim Fenn.

**Cellulosic Biofuels** Andrea Asztalos (U. Notre Dame), Giovabbi Bellesia (T10), Daewon Cho (UNM), Taraka Dale (B7), Debra Dunnaway Mariano (UNM), David Fox (B8), Gnanakaran (T10), Ken Hammel (USDA), Marcel Lucas (C-PCS), Pat Mariano (UNM), Marat Mustyakimov (B8), Norma Pawley (B8), Partha Rangaswamy (T10), Kirk Rector (C-PCS), Tongye Shen (T10), Pat Unkefer (UNM), Greg Wagner (C-PCS), Mary Jo Waltman (B8)

**Xylanases** Andrey Kovalevsky (B8), David Fox (B8)